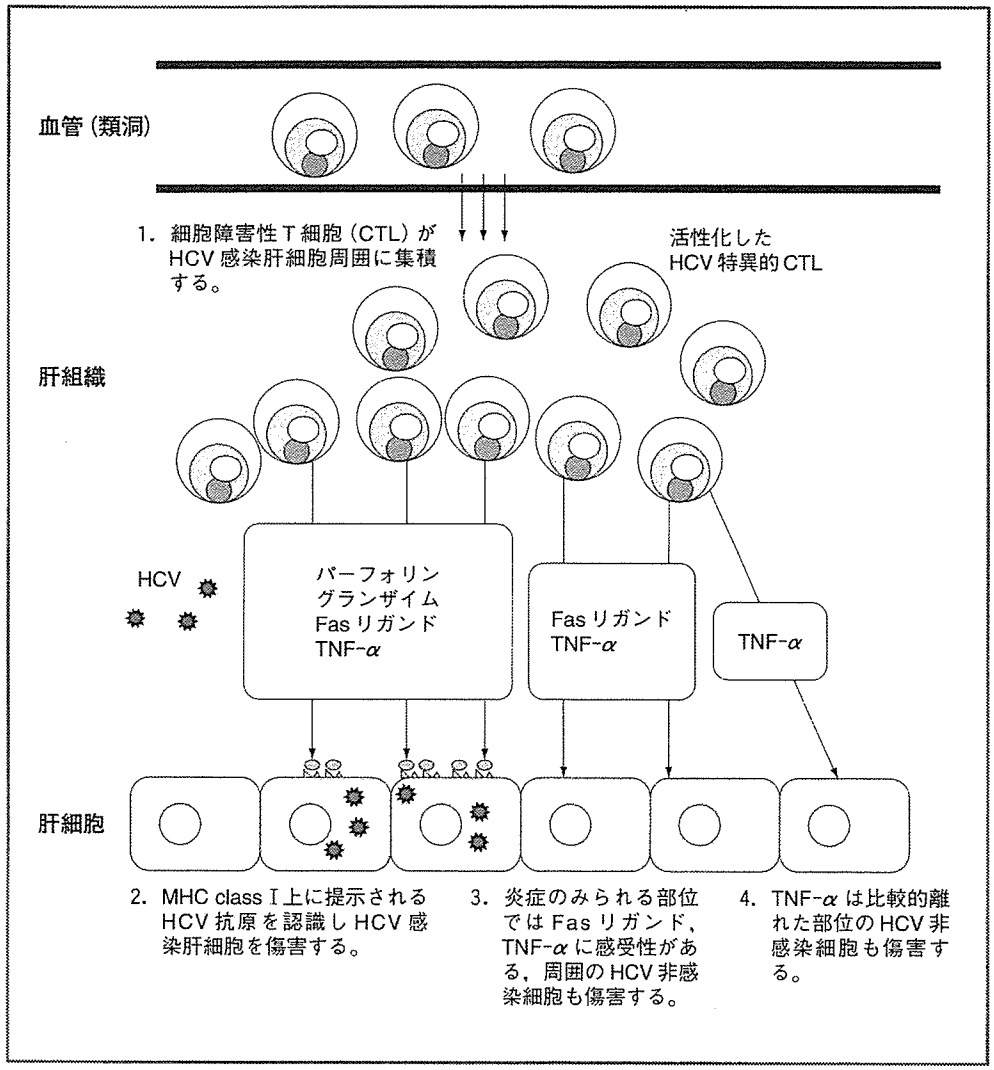

図3　HCV特異的細胞障害性T細胞による肝細胞傷害

持続感染に寄与しているとされている。しかし一方，チンパンジーでのHCV感染実験では，HVR1に変異はみられずに持続感染に発展しているとの報告もあり，E2領域の変異が感染の持続化に大きな役割を果たしているかを疑問視する意見もある。

2）細胞性免疫からの逃避

HLA class Ⅰあるいはclass Ⅱ拘束性T細胞のエピトープの変異は，HCV感染肝細胞の排除を妨げることにより感染の持続化に寄与する可能性がある。これまでの研究では，感染HCVのクローニングにより，CD8⁺CTLの認識を妨げるアミノ酸変異が認められている。また，HLA-DRB1拘束性でNS3由来のペプチドを認識するTh1タイプのT細胞に対し，このエピトープ内の1つのアミノ酸に変異が起こると，分泌するサイトカインがTh1タイプからTh2タイプに

変化したことが報告されたが[15]，このようにTh 2タイプの免疫応答が生体内で優位になると持続感染が成立しやすくなると考えられる。

C型急性肝炎6例での検討では，HCVのCTLエピトープにおけるアミノ酸変異は3例にみられ，変異した配列はもとの配列と比較し，CTLに認識されにくく，さらにCTLを効果的に誘導できないと報告された[16]。CTLエピトープの変異は，より強いエフェクター機能を有するCTLをターゲットにして起こり，持続感染に寄与することも想定される。

一方，HCV感染に対する初期のCTL応答は多様であり，1つのエピトープの変異のみで持続化は説明できないとの指摘もある。エスケープミュータントは持続感染の原因というより，持続感染の結果をみている可能性も否定できない。

2．HCV感染による免疫細胞の機能抑制

抗HCV抗体は感染後2～4ヵ月間もの間出現せず，さらに抗体が現れてもHCV感染は持続し肝炎は進行する。また，細胞性免疫においても，急性期には多様なT細胞応答が認められるが，慢性化するとその応答は劇的に減弱してしまう。肝内には多数のHCV特異的CD 8^+ T細胞が存在してはいるものの，HCVを排除できない。さらに，C型慢性肝炎患者にはB型肝炎ウイルスや細菌感染などの合併も多く，以前から生体での免疫力の低下が想定されてきた。近年，HCV自体が能動的に生体の免疫機構を抑制している可能性が考えられており，それを示唆する報告が多数なされるようになった。

1）自然免疫に対する抑制

HCVのE2蛋白は細胞表面上のCD 81と高い親和性をもち，CD 81はHCVが細胞感染する際の受容体となりうると考えられている。HCVのE2蛋白はNK細胞上に発現するCD 81と結合し，直接NK細胞の機能を低下させる作用があることが報告された[17]。NK細胞はIFN治療1週間後には肝内への浸潤が観察されるが，治療有効群と治療無効群との間でNK細胞の細胞障害活性は異なり，IFN治療の有効性を予知する指標となるとされている。このことからも，NK細胞を中心とした自然免疫系もHCV排除には重要な役割を果たすと考えられるが，HCVが直接NK細胞の活性を抑制することは感染の持続化に対し大きな意味をもつ。

NK細胞の細胞障害活性低下の具体的な機序については，いくつかの報告がなされている。HCVコア蛋白は，*p53*依存性にtransporter associated with antigen processing 1（TAP 1）の発現を増強することで，MHC class I発現を増強する。MHC class Iの発現増強は，NK細胞のHCV感染肝細胞に対する細胞障害活性を低下させ，感染の持続化に寄与する可能性がある。また，C型慢性肝炎患者のNK細胞では，その活性を抑制するシグナルを伝えるレセプターであるCD 94/NKG 2 Aの発現が増強していると報告された[18]。さらに，HLA A 2拘束性T細胞のエピトープとして知られているHCVコア蛋白35-44のアミノ酸配列に相当するペプチドは，CD 94/NKG 2 Aのリガンドであるヒト-Eと結合することによりHLA-Eの発現を安定化させて，NK細胞による障害を抑制し，HCVの持続感染に寄与する可能性も考えられている[19]。NK細胞の他の抑制性レセプターであるKIR 2 DL 3がHCVの排除に影響を及ぼすことも想定されている[20]。

2）液性免疫に対する抑制

C型肝炎患者の末梢リンパ球は，前述のCD 81分子が強発現しており，HCVが感染しやすい状態になっていることが考えられ，感染を介して抗体産生などに影響を及ぼしている可能性がある。また，他の感染実験でも，中和抗体の抗体価は低く再感染を防止することはできなかったため，B細胞応答もHCVにより抑制されていることが想

定されている。

3) T細胞に対する抑制

HCV特異的CTLのエフェクターとしての機能は明らかに低下している。C型慢性肝炎患者の末梢リンパ球においては，その機能の発現に重要なCD 3ζ鎖の発現が，また肝浸潤リンパ球においてはT細胞レセプターδ鎖やCD 56の発現が低下していることが報告されている。また，HBV特異的CTLに比しHCV特異的CTLは明らかにパーフォリンの発現量が少なく，これは機能低下を示す1つの例とされているが，このような免疫細胞自体の機能低下もC型肝炎の持続化に関与することが想定されている。C型急性肝炎時にはCCR 7$^-$ CD 8$^+$ T細胞(メモリー・エフェクター細胞)は細胞障害活性が低下しているが，これにIL-2を加えると完全にエフェクター機能を有する細胞になることから，T細胞が活性化する際のIL-2の欠乏がCTLの機能低下の主な原因であるとの報告もある[21]。循環しているコア蛋白がIL-2産生のシグナル伝達の抑制に関与していることも想定されている。

C型慢性肝炎患者において，HCV特異的CD 4$^+$ T細胞の存在は認められるものの抗原特異的な増殖能は抑制され，さらに後述するように，抗原刺激に特異的なIL-10やTGF-β産生も有意に認められており，Th細胞やCTLの十分な活性化が起こらずにHCVの持続感染を導くと考えられる。

また，HCV感染肝細胞より遊離し末梢血中に存在するHCVコア蛋白は，T細胞のgC 1 qRと結合することで，T細胞の増殖や活性，IFN-γ産生能を阻害することが報告された。HCVコア蛋白は血中にナノグラムの単位で存在しており，gC 1 qRと結合するには十分量と考えられるが，肝組織内ではさらに高濃度のコア蛋白が存在していると想定され，肝浸潤リンパ球に少なからず影響を与えていると推測される。

HCV NS 4 A/B蛋白は，細胞内で小胞体からゴルジ体への輸送を妨げることにより，HLA class I分子の細胞上への発現を抑制することが報告された[22]。これにより，HCV特異的CD 8$^+$ T細胞がHCV感染肝細胞を認識しにくくなり，HCVの感染持続化に繋がることも考えられる。

さらに，肝臓には類洞内皮細胞やKupffer細胞といった免疫に関与する細胞が存在するが，それらは成熟した樹状細胞とは異なり，ウイルス抗原は提示するもののCD 80やCD 86といった共刺激分子に乏しいため，T細胞を十分に刺激できないばかりか，かえって免疫寛容を誘導してしまうことも考えられている[23]。

4) 樹状細胞に対する抑制

樹状細胞は免疫を誘導するうえで，重要な役割を担っている。C型慢性肝炎患者においては，樹状細胞のallogeneicのT細胞を刺激する能力が低下していることや，HCVコアとE 1蛋白は樹状細胞の成熟化を抑制することでT細胞刺激能を減弱させていたことがこれまでに指摘されている。近年，C型慢性肝炎患者では形質細胞様樹状細胞の数やIFN-α産生能はいずれも低下し，一方，骨髄系樹状細胞ではfrequencyに差異は認められないものの，アロリンパ球の刺激能は低下していることが報告された[24]。この報告では，骨髄系樹状細胞の機能低下はHCVコア蛋白とNS 3蛋白で誘導可能であり，これらの蛋白の認識はTLR 2を介して行われるとされた。これは，ウイルス蛋白が樹状細胞の機能を低下させ，細胞免疫応答の誘導を抑制する1つのメカニズムになると考えられる。また，細菌やウイルス由来のTLRリガンド(病原体成分)により活性化した樹状細胞は，クロスプレゼンテーションの作用が弱まり，免疫応答が減弱することが報告されたが[27]，HCVにも同様の機構が存在していることも考えられる。実際，C型慢性肝炎患者の未成熟の樹状細胞では，健常者と比較してTLR 2の発現が低

下しており，TLR 2で刺激した樹状細胞はT細胞増殖効果が減弱していることも示された[28]。

樹状細胞によるNK細胞活性化の抑制も指摘されている。IFN-α刺激後に，樹状細胞はその表面にMHC class I-related chain A and B (MICA/B)を発現させ，NK細胞を活性化するが，C型慢性肝炎患者においてNK細胞が有効に活性化できないのは，type I IFNによるIL-15産生能が低下しており，MICA/B発現の増強が抑制されていることが原因であるとされた[25]。

HCVは，E 2蛋白が樹状細胞上に発現するDC-SIGNに結合することより樹状細胞にも感染することや，soluble E 2蛋白も樹状細胞と結合が可能であることも報告され，それらにより樹状細胞は機能低下に陥る可能性も考えられている。HCVコア，NS 3，NS 5 A，NS 5 B蛋白は成熟した樹状細胞にアポトーシスを誘導することも報告されている[26]。

一方，チンパンジーへの感染実験では，樹状細胞の機能低下は認められないとする結果や，C型慢性肝炎患者の検討で樹状細胞の成熟化やアロ刺激能は正常であることも報告されている[29]。HCVコアやNS 3蛋白を発現させたヒト樹状細胞は，炎症性サイトカインの産生やフェノタイプ，アロT細胞刺激能は正常であるとされた[30]。健常者と比較してC型肝炎患者では形質細胞様，骨髄系とも樹状細胞のfrequencyは低いが，循環血中の樹状細胞のフェノタイプや機能は低下しておらず，形質細胞様樹状細胞においてIFN-α産生能も1つひとつの細胞レベルでは低下していないとの報告もある[31]。また他の報告によると，HCVの急性肝炎では，末梢血中に形質細胞様樹状細胞が著明に減少しているが，それらは未成熟でHLA DRやCCR 7の発現やIFN-αの産生能が低下しているとする一方，慢性肝炎患者の樹状細胞では，形質細胞様樹状細胞に明確な変化は認められていない[32]。

樹状細胞のC型肝炎への関与は今後さらなる検討が必要である。

5) HCV蛋白による細胞に対するその他の影響

HCVトランスジェニックマウスの系で，HCV蛋白がIFNによりもたらされる細胞内伝達シグナル(Jak-STAT系)を抑制することが示唆され，これがIFN不応性の一因となることが想定されている[33]。また，HCV蛋白は感染した肝細胞のFasを介したアポトーシスを抑制しており，これも持続感染を誘導するのに重要であると指摘している。一方，HCVコア蛋白はJurkat細胞に対しFasを介したアポトーシスの経路を活性化させるとの報告もある[34]。細胞内コア蛋白は，TNFレセプターの細胞内ドメイン(TNFRI)あるいはFasと結合し，細胞にアポトーシスを誘導することが証明され，肝細胞やリンパ球のアポトーシスに関与する可能性も考えられている。

3．HCV特異的制御性T細胞の関与

生体の免疫応答を抑制する要因の1つとして，抗原を特異的に認識してIL-10やTGF-βを産生する制御性T (regulatory T ： Tr)細胞が注目されている。C型慢性肝炎患者においては，Tr細胞と考えられる$CD\,4^+\,CD\,25^+$ T細胞のfrequencyが高く，この細胞集団は直接T細胞の機能を抑制し，これがHCV特異的細胞性免疫の質的，量的な抑制を引き起こして，肝炎の持続化に寄与していると想定されている[35](図4)。HCVコア蛋白に特異的なTr細胞がC型慢性肝炎の末梢血から分離誘導され，この細胞が産生するIL-10がHCV感染の持続化に関与すると報告された[36]。また，C型肝炎患者の肝内にはIL-10を産生する$CCR\,7^-\,CD\,8^+$ Tr細胞が存在し，肝内に多数集積している$CCR\,7^-\,CD\,8^+$メモリーT細胞の機能を抑制することも報告された[37]。さらに，HCV NS 4蛋白は，C型肝炎患者のみならず，健常者の末梢単核球からもIL-10の産生を促しIL-12の分泌を抑制させ，さらに樹

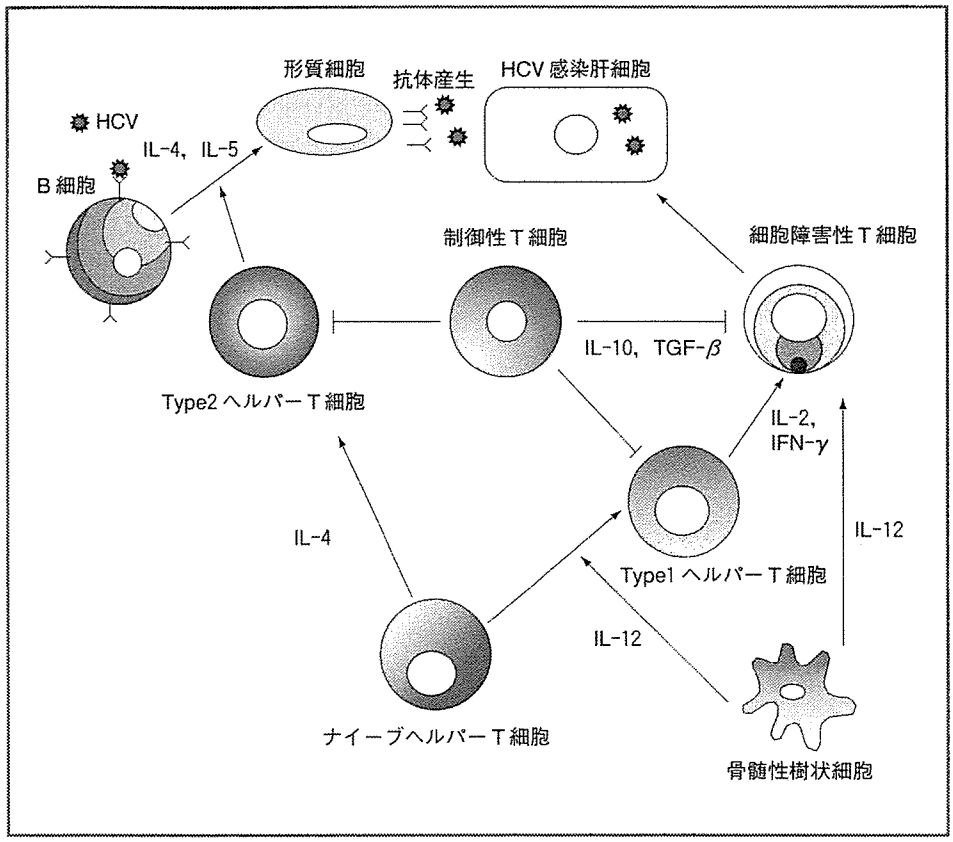

図 4　HCV に対する免疫細胞の誘導と制御性 T 細胞による免疫抑制

状細胞の分化成熟化を抑制するとの報告もあり，細胞性免疫の活性化抑制の 1 つの機序として興味深い[38]。

以上のように Tr 細胞の HCV 感染持続化への関与が強く示唆されているが，まだ不明な点も多くさらなる検討が必要である。

4．その他の HCV 持続感染の機序

非構造蛋白領域である NS 5 A の C 末端側に存在する 40 個のアミノ酸からなる部分(IFN-sensitivity determining region)の変異は，IFN により誘導され，抗ウイルス効果を発揮するプロテインキナーゼと結合し，その活性化を阻害することにより IFN 治療抵抗性を得るとされている。また，HCV NS 3/4 のセリンプロテアーゼは，細胞が抗ウイルス効果を発揮するうえで重要な interferon regulatory factor-3 を抑制するといわれている。近年，HCV NS 3/4 のセリンプロテアーゼは，二本鎖 DNA の受容体である retinoic acid inducible gene I(RIG-I)と抗ウイルスシグナルとを結ぶアダプター蛋白質である Cardif を標的とし，これを不活化させることにより細胞の抗ウイルス効果を抑制していることが報告された[39]。また，分子レベルではウイルスが感染細胞に，ウイルス遺伝子発現抑制，抗原のプロセッシング抑制といった影響を与えていることも想定されている。

HCV 感染に対する免疫を介した治療

IFN-α は現在，HCV を生体から排除すること

が可能な唯一の治療薬である。低濃度のIFN-αは，HCVコア蛋白をターゲットにしたDNAワクチンにより誘導される細胞性免疫応答を3～4倍増強するが，高濃度では逆に，CTL応答を抑制すると報告された[40]。C型肝炎治癒症例はTh 1優位であるといわれているが，近年，C型慢性肝炎の治療に用いられているリバビリン(商品名レベトール®)は，患者の免疫応答をTh 2からTh 1優位に変化させることが，抗ウイルス効果の1つの機序と考えられている。

IFN治療によりSVRが得られた症例ではRelapse例や無効例に比し，肝内や末梢のHCV特異的CTL活性が強くみられることも報告されている[41]。このなかで筆者らは，IFNとリバビリンの併用療法は，CTL応答を増強してHCVを排除するというより，治療前から存在するCTL応答がIFN・リバビリン併用療法による直接の抗ウイルス作用を増強して排除するのではないかと述べている。

樹状細胞にNS 3蛋白をパルスし，CpGオリゴヌクレオチドで成熟させるとCD 40の発現が増強し，強い免疫応答を誘導することができ，マウスでHCV NS 3を発現させたワクシニアウイルスの感染を抑制できたことから[42]，樹状細胞を用いた細胞免疫療法の開発も今後期待される。

おわりに

肝炎動物モデルや臨床検体の解析などにより，ウイルス肝炎における免疫応答が長年にわたり研究されてきた。肝細胞障害にはCTLを中心とした細胞性免疫応答の関与が明らかになり，その障害機序や生体免疫応答の抑制機序も徐々に解明されてきている。免疫応答を適切にコントロールすることは，肝炎ウイルス排除あるいは肝炎の鎮静化に重要であるが，これから解明していかなければならない問題も多々ある。これらの問題点を生体免疫反応のみならず，ウイルス側からも詳細に解明していくことで，将来，肝炎ウイルスを完全に生体から排除できる治療法の開発が可能になると考えられる。今後のウイルス学，免疫学のさらなる発展を期待する。

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Viral covalently closed circular DNA in a non-transgenic mouse model for chronic hepatitis B virus replication

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***Background/Aims*: The lack of small animal models supporting chronic hepatitis B virus (HBV) infection impedes the assessment of anti-viral drugs in the whole animal. Although transgenic mice have been used for this purpose, these models are clearly different from natural infection, because HBV is produced from the integrated HBV sequence harbored in all hepatocytes.**

***Methods*: Balb/cA nude mice were hydrodynamically injected with a plasmid having 1.5-fold over-length of HBV DNA and analyzed for HBV replication.**

***Results*: Hydrodynamically injected mice showed substantial levels of antigenemia and viremia for more than 1 year. Covalently closed circular DNA (cccDNA), the template of viral replication in natural infection, was produced in the livers and was critically involved in the long-term HBV production, because disruption of the *pol* gene of the inoculated DNA resulted in transient expression of HBV genes for less than 2 months. Administration of the IFNα gene transiently suppressed HBV DNA replication, but was not capable of eliminating HBV in this model.**

***Conclusions*: In vivo gene transfer of a plasmid encoding HBV DNA can establish chronic viral replication in mice, which involves, at least in part, new synthesis of the HBV cccDNA episome, thus recapitulating a part of human HBV infection.**

Keywords: **HBV; Liver; DNA; Hydrodynamics; Naked; Gene; Transfer; Transfection; IFN; Mouse**

1. Introduction

Hepatitis B virus (HBV) causes both transient and persistent infection in the human liver [1,2] When healthy adults are exposed to this virus, they usually develop acute transient infection with various degrees of liver injury, and, in most cases, have favorable outcomes. In contrast, when immunocompromised hosts such as newborn babies, drug abusers, and patients receiving immunosuppressive drugs, are infected with HBV, they cannot eliminate it and often suffer from chronic liver injury and hepatocellular carcinoma. Chronic carriage of this virus is a major health problem in many countries. Patients with chronic HBV infection are currently treated with interferon (IFN) or nucleotide analogs such as lamivudine and adefovir. However, the limited success and frequent recurrence after cessation of therapy require new strategies for terminating this viral infection.

Study of HBV replication in vivo is hampered by the lack of suitable small and well-characterized animal models; thus far, only chimpanzees and the tree shrew (Tupaia), a relatively uncharacterized animal, appear to support HBV infection [3]. Several lines of transgenic mice have been established but HBV replication is generated from the integrated HBV sequence harbored in all hepatocytes, which is clearly different from the natural infection [4,5]. An alternative strategy is in vivo gene transfer of HBV DNA. Takahashi et al. [6] previously reported that

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intrahepatic injection of naked HBV DNA with cationic liposome can cross the species barrier and leads to HBV replication in rats. We and others have reported that hydrodynamics-based delivery of HBV DNA efficiently transduces murine livers and leads to HBV replication [7,8]. However, HBV replication in these models is terminated within a couple of weeks, presumably resulting from immunological elimination of HBV-expressing hepatocytes. Very recently, there have been reports of these models being applied for the assessment of anti-viral drugs [9–11]. However, the analysis may be hampered because this is a model of acute transient infection and would not allow observation of the long-term outcome.

In an attempt to develop a better long-term model, we hydrodynamically injected a plasmid encoding replication competent HBV DNA into immunocompromised mice and examined the kinetics of expression and replication of HBV. The mice produced HBV-related proteins for over 1 year, which appeared to be dependent on episomal HBV DNA replication in the liver, because the introduction of replication-incompetent HBV DNA led to transient expression of HBV genes. IFNα treatment of these mice showed transient repression of HBV replication but could not terminate it. These mice mimic a part of human HBV infection in terms of the template of viral replication and should be useful for analyzing the long-term outcome of anti-HBV therapy.

2. Materials and methods

2.1. Plasmids and mutagenesis

Plasmid pHBV1.5 containing an overlength (1.5-mer) copy of HBV DNA (GenBank accession no. AF305422) has been described previously [7]. A plasmid containing mutant HBV DNA carrying a stop codon instead of 54Trp of the *pol* gene was generated from pHBV1.5 by a GeneTailor Site-Directed Mutagenesis system (Invitrogen, Carlsbad, CA) and verified by sequencing. The site of the mutation was designed not to affect the expression of any HBV-related genes except for the *pol* gene. A plasmid coding the murine IFNa1 gene, pCMV-IFNa1, was generously provided by Dr Daniel J.J. Carr (University of Oklahoma, Health Science Center) [12].

2.2. Mice

Specific pathogen-free female Balb/cA nude mice were purchased from Clea Japan, Inc. (Tokyo, Japan) and were used at the age of 5 to 6 weeks. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care and study protocol complied with the institution's guideline.

2.3. Injection of naked plasmid DNA

Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Hydrodynamic injection of plasmid DNA was performed according to previous reports [13,14]. In brief, 25 μg of plasmid DNA was diluted with 2.0 ml of lactated Ringer's solution and injected into the tail vein using a syringe with a 30-gauge needle. DNA injection was completed within 8 to 15 s.

2.4. Nothern blot

Total tissue RNA was isolated with Isogen (Nippon Gene, Toyama, Japan), and then 30 μg of total RNA was analyzed by Northern blotting with the HBV adw2 probe, as described previously [15].

2.5. Immunohistochemistry

For immunohistochemical detection of HBc protein, tissues were fixed with 10% neutral buffered formalin and embedded in paraffin. After being deparaffinized, sections 4 μm thick were incubated with anti-HBc antibody (Dako, Denmark), followed by immunoperoxidase staining using the ABC procedure (Vector Laboratories, Burlingame, CA) and counterstaining with hematoxylin.

2.6. Detection of hepatitis B antigens in serum

Under light anesthesia using sevoflurane, animals were bled from the retro-orbital vessels. Serum HBs antigen and HBe antigen were measured by chemiluminescent immunoassay (CLIA system, Abbott Laboratories, North Chicago, IL).

2.7. Real-time detection of HBV DNA in serum

Serum was treated with DNase I (Takara, Tokyo, Japan) and then proteinase K. DNA was extracted from the sera by a QIAamp DNA blood isolation system (Qiagen). HBV DNA was quantified by using real-time polymerase chain reaction (PCR) technology (Applied Biosystems, Foster City, CA) as described previously [16]. Primers and fluorescent probes are as follows: sense (nucleotides 168–188), 5′-CACATCAGGATTCCTAGGACC-3′; antisense (nucleotides 341–321), 5′-GGTGAGTGATTGGAGGTTGG-3′; probe (nucleotides 244–269), 5′-FAM-CAGAGTCTAGACTCGTGGTGGACTTC-3′.

2.8. Density analysis of HBV particles in serum

DNase I-treated serum was clarified by centrifugation at 15,000 rpm for 15 min using a 0.45 μm membrane filter. The clarified serum was layered on top of a 10–60% discontinuous sucrose gradient. Centrifugation was carried out at 141,000 *g* for 48 h. Fractions were collected from the bottom of the tube. After treatment with proteinase K, DNA was isolated from each fraction and applied for analysis of HBV DNA by PCR [7]. In an additional experiment, DNase I-treated serum was incubated with 1% Nonidet P-40 and 0.3% 2-mercaptoethanol for 16 h at 37 °C, and then used for density analysis.

2.9. Detection of HBV covalently closed circular DNA (cccDNA)

DNA was isolated from liver tissues by using a DNeasy Tissue kit (Qiagen). PCR detection of cccDNA was performed according to the procedure of Jun-Bin et al. [17] with some modification (Fig. 1). The PCR product was analyzed on a 1.2% agarose gel by electrophoresis. In some experiments, cccDNA was quantified using real-time PCR. To calculate the number of cccDNA per HBcAg-positeve hepatocyte, the total number of hepatocytes was estimated from the genomic DNA content in the murine liver under the assumption that the liver is about 70% hepatocytes. In addition, ampicillin resistance gene in the plasmids was amplified by using a sense primer (5′-TATGGCTTCATTCAGCTCCG-3′) and an antisense primer (5′-TCGAACTGGATCTCAACAGC-3′).

2.10. IFNα gene therapy

At 70 days after pHBV1.5 injection, nude mice were hydrodynamically injected with either pCMV-IFNa1 or pCMV mock plasmid and examined

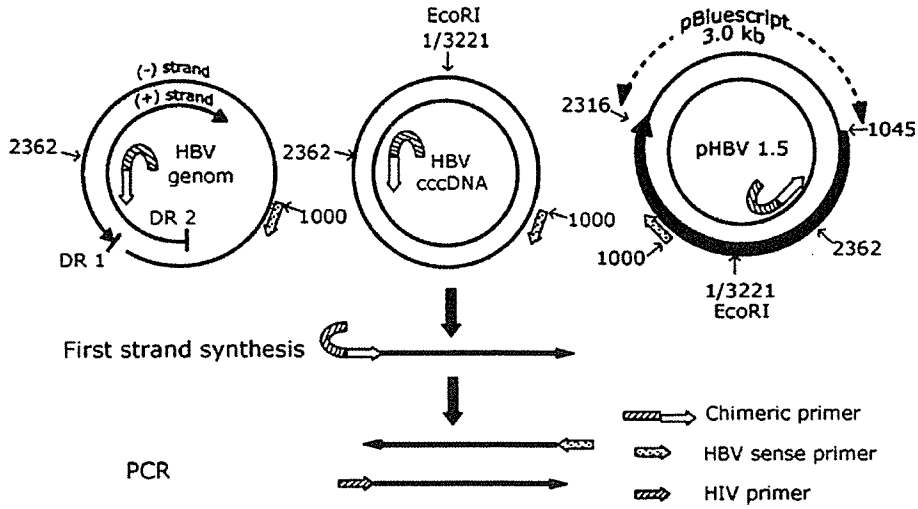

Fig. 1. Principle of PCR detection of HBV cccDNA. Three forms of HBV sequences which could have been present in our samples are shown: HBV genome, HBV cccDNA, and pHBV1.5. The number of nucleotides starts at the EcoRI site. A chimeric primer (5′-TCGCTTTCGGGTCCCTGGTCCCGTCGTC-3′) is composed of two segments: the segment A sequence near the 5′ end is HIV-specific and the segment B sequence near the 3′ end is complementary to the HBV DNA plus strand from nucleotide 2362 to 2351. With DNA polymerase activity, the chimeric primer extends and produces a new single DNA strand. Since the HBV plus strand has a gap, nucleotide extension will be stopped at the DR2 gap. On the other hand, an extremely long strand will be generated if pHBV1.5 acts as a template DNA. One-twentieth volume of the elongated strand was used as a template in the next PCR amplification in the presence of one primer, identical to the chimeric primer segment A (HIV primer; 5′-TCGCTTTCGGGTCCCT-3′) and another primer complementary to the HBV DNA minus strand from nucleotide 1000 to 1016 (HBV sense primer 5′-TTGTGGGTCTTTTGGG-3′), cycled 35 times through a program of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min. The 1352 bp products will be amplified only from cccDNA. In real-time PCR, the DNA samples were digested with EcoRI and ScaI and subjected to the elongation reaction followed by PCR using a fluorescent probe (5′-FAM-GAGACCACCGTGAACGCCCATCAGAT-3′ (nucleotides 1444–1469)). ScaI site is located in the ampicillin resistance gene of the pBluescript but not in HBV DNA sequence.

for HBV replication. IFNα production was assessed using a commercially available mouse IFNα ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ).

3. Results

3.1. Intravenous injection of pHBV1.5 leads to hepatitis B antigenemia as well as hepatic expression of HBcAg for more than 1 year

We injected 25 μg of pHBV1.5, which contains 1.5-fold overlength HBV DNA, into the tail veins of nude mice with acute circulatory overload. To investigate the expression of HBV, the presence of HBV transcripts was analyzed by Northern blot in various organs from mice sacrificed at 3 days after the injection (Fig. 2A). Two major bands corresponding to 3.5 and 2.4/2.1 kb transcripts were detected in the liver but not in other tissues including the kidney, spleen, thymus, lung, heart, and brain. The levels of HBsAg and HBeAg in the serum were serially determined by a quantitative CLIA method (Fig. 2B). Although the levels of HBsAg rapidly decreased 1.5 log within the first 2 weeks, all mice were persistently positive for HBsAg and HBeAg for more than 1 year. Immunohistochemical analysis revealed that around 4% of the hepatocytes were positive for HBc at 3 days after injection (Fig. 2C). HBcAg-positive cells gradually decreased in number but were still detected at one year after the injection (Fig. 2D). Although data are not shown, hepatic damage could not be detected, as evidenced by biochemical and histological analysis, throughout the course, except during the first week; it resulted from hemorrhagic destruction of the liver due to hydrodynamic pressure. Taken together, these results indicated that hydrodynamics-based delivery of a plasmid encoding replication-competent HBV DNA can establish specific expression of HBV genes in the liver and persistent expression without significant liver injury for a period of more than 1 year.

3.2. Long-term productive replication of HBV DNA

To examine if viral particles are produced into the circulation, sera obtained at 3 days after pHBV1.5 injection was treated with DNase I and fractionated by sucrose density gradient centrifugation. As shown in Fig. 3A, when each fraction was assayed in PCR for the presence of HBV DNA, the strongest signal was observed in the fraction with a density of 1.21 g/ml, corresponding to the density of HBV particles derived from human sera [18]. In addition, when serum was pre-treated with detergent before the centrifugation, the positive fraction shifted to a density of 1.28 g/ml, suggesting that detergent treatment releases core particles from HBV particles by removing the envelope.

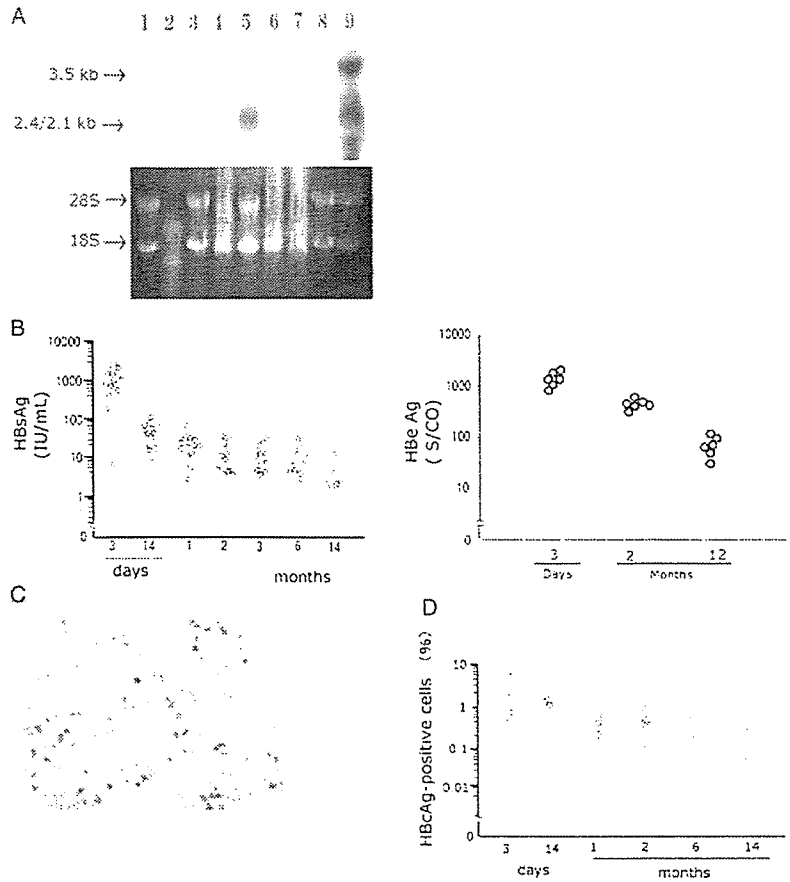

Fig. 2. Expression of HBV in hydrodynamically-transfected nude mice. (A) HBV RNA expression in various organs. Total RNA isolated from the indicated organs was analyzed for the presence of HBV-specific sequence 3 days after transfection (upper panel). Huh7 transfected with pHBV1.5 or pCMV were included as positive and negative controls, respectively. Arrows indicate 3.5- and 2.4/2.1-kb transcripts. A photograph of the ethidium bromide-stained gel is also shown in the lower panel. Lane 1, brain; lane 2, heart; lane 3, thymus; lane 4, lung; lane 5, liver; lane 6, kidney; lane 7, spleen; lane 8, pCMV-transfected Huh7; lane 9, pHBV1.5-transfected Huh7. (B) HBsAg and HBeAg in serum. The levels of HBsAg and HBeAg were serially determined in a cohort of mice hydrodynamically transfected with pHBV1.5. (C) Immunohistochemical detection of HBc Ag. Representative data for nude mice 3 days after pHBV1.5 injection. (D) Frequency of HBcAg-positive hepatocytes in the livers.

To examine the kinetics of viremia, we examined the levels of DNase I-resistant HBV DNA in serum by real-time PCR analysis (Fig. 3B). The levels of HBV DNA were as high as 1×10^7 copies/ml at 3 days after the injection and gradually decreased by 1.5 log over 1 year.

3.3. Long-term expression of HBV is dependent on HBV replication

The extremely long-term expression and carriage of HBV in this system led us to examine whether episomal replication could affect the kinetics of expression of HBV-related genes. Toward this goal, we introduced point mutation in the *pol* gene of pHBV1.5 which could produce the truncated form of the HBV polymerase without affecting the expression of any other HBV-related proteins. Mice hydrodynamically injected with mutant pHBV1.5 produced HBsAg as well as HBcAg at levels similar to those of wild-type pHBV1.5-injected mice 3 days after injection (Fig. 4A and B). However, mutant pHBV1.5-induced expression of HBsAg, HBeAg and HBcAg was terminated within 2 months, in striking contrast to wild-type pHBV1.5-induced gene expression (Fig. 1B and D). Northern blot analysis confirmed the transient expression of HBV genes after injection of mutant pHBV1.5 (Fig. 4C).

HBV DNA polymerase binds to the 5′ end of its own mRNA template, and the complex is then packaged into nucleocapsids, where viral DNA synthesis occurs [19]. HBV genomic DNA produced via the reverse transcription pathway predominantly consists of relaxed-circular DNA with a complete minus strand and a partially synthesized plus strand. In natural HBV infection in humans, part of the nucleocapsids migrates to the nucleus where relaxed-circular DNA is converted to cccDNA that serves as a template for transcription [19]. The finding in the present model of long-term expression of HBV involving HBV DNA replication suggested that viral cccDNA may be produced in murine livers and work as a transcriptional

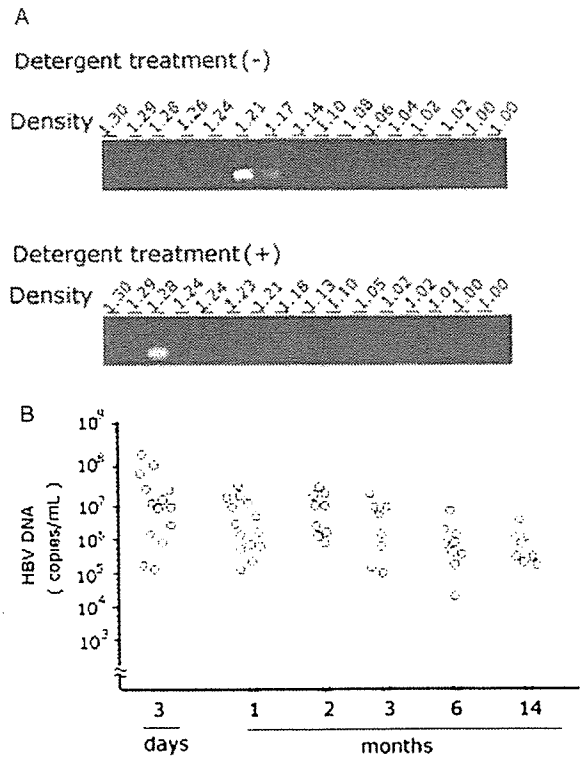

Fig. 3. Production of HBV in hydrodynamically-transfected mice. (A) Density of HBV. Sera from mice injected 3 days earlier with pHBV1.5 were treated with or without *detergent* then fractionated through sucrose density gradient. DNA was isolated from each fraction after treatment with DNase I and then the presence of the HBV DNA sequence was checked by PCR. The density (g/cm^3) of each fraction is indicated above each lane. (B) Viral titers in serum. The levels of HBV DNA were serially determined by real-time PCR.

template for HBV expression, in addition to the inoculated plasmid. To examine the presence of cccDNA in the liver, we used a PCR procedure which selectively detects cccDNA (Fig. 1). We also checked for the presence of inoculated plasmids by amplifying the ampicillin resistance gene by PCR. The authenticity of the cccDNA detection was confirmed by the detection of a specific signal from liver tissues of patients with chronic hepatitis B, but not from the serum of patients or pHBV1.5 (Fig. 5A). Viral cccDNA was clearly detected in wild-type pHBV1.5-injected livers at 3 days as well as 3 months after the injection (Fig. 5B). As expected, cccDNA was not detected in mutant pHBV1.5-injected livers. The levels of cccDNA were measured by real-time PCR (n=5 for each time point) and results were 2.4×10^7 and 6.0×10^5 copies per gram of liver tissue at 3 days and 2 months after the injection, respectively. Since the liver approximately contains 1.1×10^8 of hepatocytes, the average copy numbers of HBV cccDNA per core Ag-positive hepatocyte could be estimated to be 1 or 4. Ampicillin resistance gene was similarly amplified from both wild-type pHBV1.5- and mutant pHBV1.5-injected livers. The fact that HBV gene expression was terminated within 2 months upon injection of mutant pHBV1.5 clearly indicates that the presence of residual plasmids in the livers at later time points is not sufficient for the expression of detectable levels of HBV genes; this is consistent with a previous report [20] demonstrating that transgene expression is rapidly terminated after hydrodynamic gene delivery despite the persistence of plasmid DNA in the livers. These results support the idea that viral cccDNA is critically involved in the long-term expression and carriage of HBV in this model.

3.4. Administration of IFNα gene transiently suppressed HBV DNA replication and failed to eradicate viral template

We next sought to examine the potential usefulness of this model for the assessment of anti-viral drugs. To examine the effect of IFNα in the phase of cccDNA-dependent HBV replication, we injected either pCMV-IFNa1 or pCMV at 70 days after pHBV1.5 injection. Injection of pCMV-IFNa1 led to substantial IFNα production at day 1 (Fig. 6A), although IFNα could not be detected in the mock-injected mice (data not shown). The levels of IFNα after pCMV-IFNa1 injection rapidly declined at day 3 and could not be detected at day 28. Injection of pCMV-IFNa1 significantly suppressed viral production at day 3 but did not affect HBs production (Fig. 6B and C); this is consistent with previous findings [15,21] that IFNα suppressed HBV replication at a step of reverse transcription. In spite of the substantial suppression of HBV production at day 3, the levels of viral titers of mice injected with pCMV-IFNa1 increased to levels similar to those of pCMV-injected mice at day 14 and later. These results indicate that IFN treatment substantially suppressed viral replication, but could not eliminate the viral template from the infected host. This model should be useful for assessing anti-viral therapy aimed at eradication of the viral template.

4. Discussion

In the present study, we demonstrated that hydrodynamic injection of a plasmid encoding an overlength of HBV DNA into nude mice established long-term replication of HBV in the liver. Since hepatic damage was not observed, this model mimics the chronic carrier-like state of human HBV infections. This model reminds us of a 1988 report by Feitelson et al., [22] in which they stated that intrahepatic injection of replication competent HBV DNA led to persistent HBs antigenemia as well as chronic liver injury in nude mice. They had no evidence of HBV replication such as production of Dane particles in the circulation. In a preliminary experiment, we intrahepatically injected pHBV1.5 into nude mice and monitored viral production in the serum. DNase I-resistant HBV DNA could not be detected in most mice tested; a small number of mice

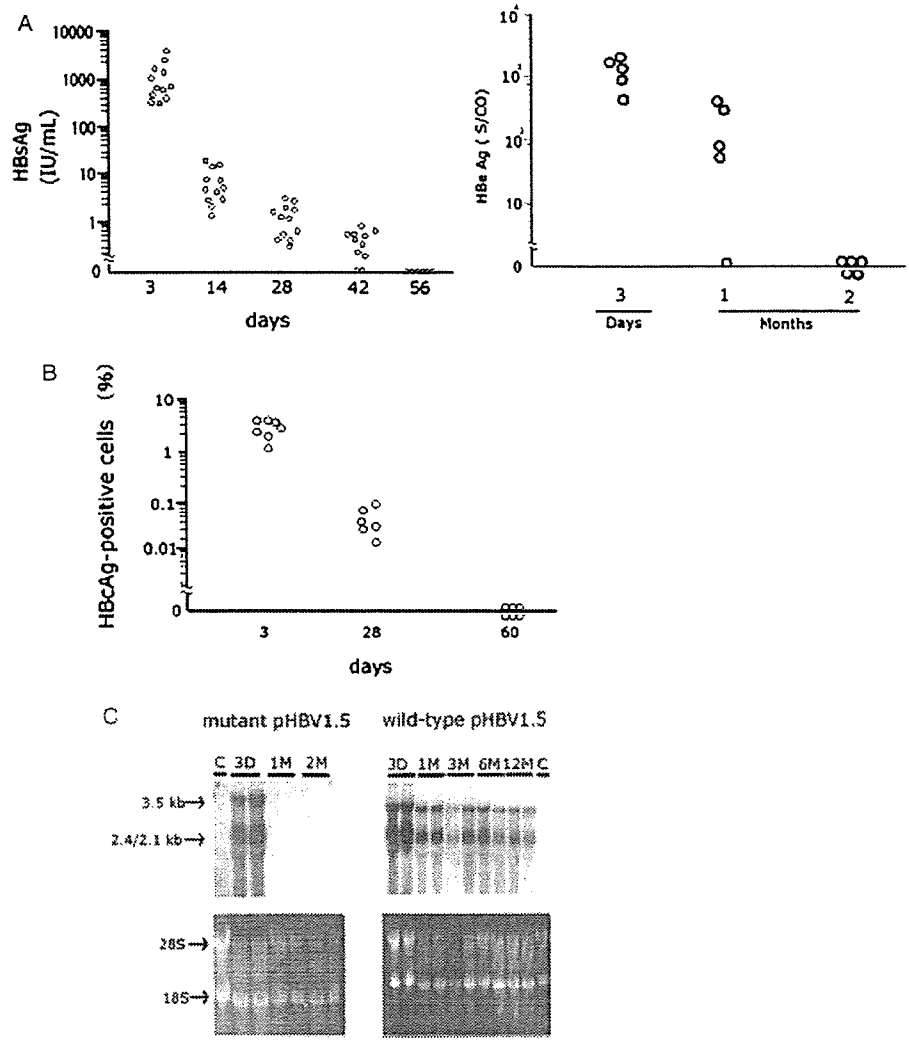

Fig. 4. Expression of HBV genes in nude mice injected with replication-incompetent pHBV1.5. (A) Serial detection of HBsAg and HBeAg in serum. (B) Frequency of HBcAg-positive hepatocytes in the livers. (C) Time-course of HBV RNA expression in the livers transfected with mutant pHBV1.5 or wild-type pHBV1.5 determined by Northern blot. C, control livers; 3D, 1M, 3M, 6M, and 12M, liver samples obtained at 3 days, 1 month, 3 , 6 , and 12 months after hydrodynamic injection, respectively.

produced low levels of virus at 3 days after injection but not at later time points (our unpublished data). Thus, the transfection efficiency of hydrodynamic injection of HBV DNA appeared to be higher than that of intrahepatic injection. Despite the difference in liver damage observed among these studies, we considered the absence of hepatitis in the present model reasonable, since the T-cell immune response towards HBV-related antigens could not occur. Furthermore, it should be noted that the antigenemia as well as HBV production achieved by the hydrodynamic procedure was very reproducible, which is critically important when applying this model to evaluate the efficacy of anti-viral drugs.

The duration of hydrodynamics-based gene expression varies among reports from days to months [19,23,24]. The plasmid-based gene expression of our model terminated within 2 months, as demonstrated by the injection of replication-incompetent HBV DNA (mutant pHBV1.5). Replication-competent HBV DNA (wild-type pHBV1.5)-injected mice displayed a rapid decline of HBsAg production followed by relatively stable antigenemia for more than 1 year (Fig. 2B). Although the rapid decline observed in the first 2 weeks may reflect the plasmid-based gene expression, stable expression of HBsAg at later time points did not depend on residual plasmids in the livers, but required intracellular reproduction of HBV DNA. These results indicate that HBV replication in addition to immunological tolerance is critically important for long-term HBV expression in this system. Previous research on in vivo gene transfer [8] and transgenic mice [5] has indicated that HBV cccDNA, the template of HBV replication in natural infection, could not be detected in

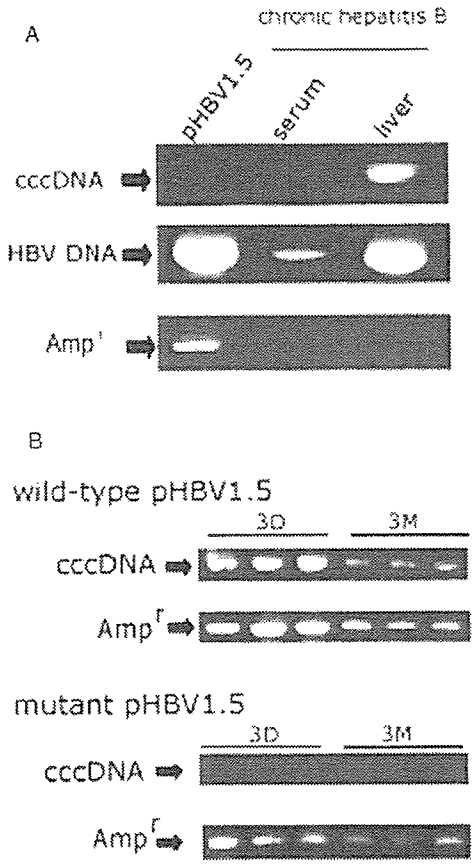

Fig. 5. Detection of HBV cccDNA and ampicilin resistance gene by PCR. (A) Specificity of HBV cccDNA detection by PCR. DNA were isolated from the following samples and amplified for the detection of cccDNA, ampicilin resistance gene, and HBV DNA sequence by corresponding PCRs. pHBV1.5, sample containing pHBV1.5; serum, serum from a patient with chronic hepatitis B; liver, a liver specimen from the same patient. (B) DNA was isolated from the liver samples 3 days (3D) or 3 months (3 M) after hydrodynamic injection of either wild-type pHBV1.5 or mutant pHBV1.5 ($N=3$ for each group) and examined the presence of cccDNA and ampicilin resistance gene by PCRs.

murine livers by Southern blot analysis. In the present study, we applied a highly sensitive PCR procedure and detected HBV cccDNA in pHBV1.5-injected livers. What is important is that the estimated numbers of HBV cccDNA per hepatocyte were 1 or 4, which should be sufficient for HBV gene expression. Taken together, the present study is the first demonstration of the production of viral cccDNA and its contribution to HBV replication in mice. Thus, the species restriction on the production of HBV cccDNA may not be as strict as has previously been believed.

Mutation of HBV DNA occurring during therapy with various nucleotide analogues leads to drug resistance and limits the success of these drugs for controlling HBV replication in humans [1,2]. Thus far, except for the in vitro recombinant HBV baculovirus system [25], there has been no useful model supporting reproduction of the HBV viral

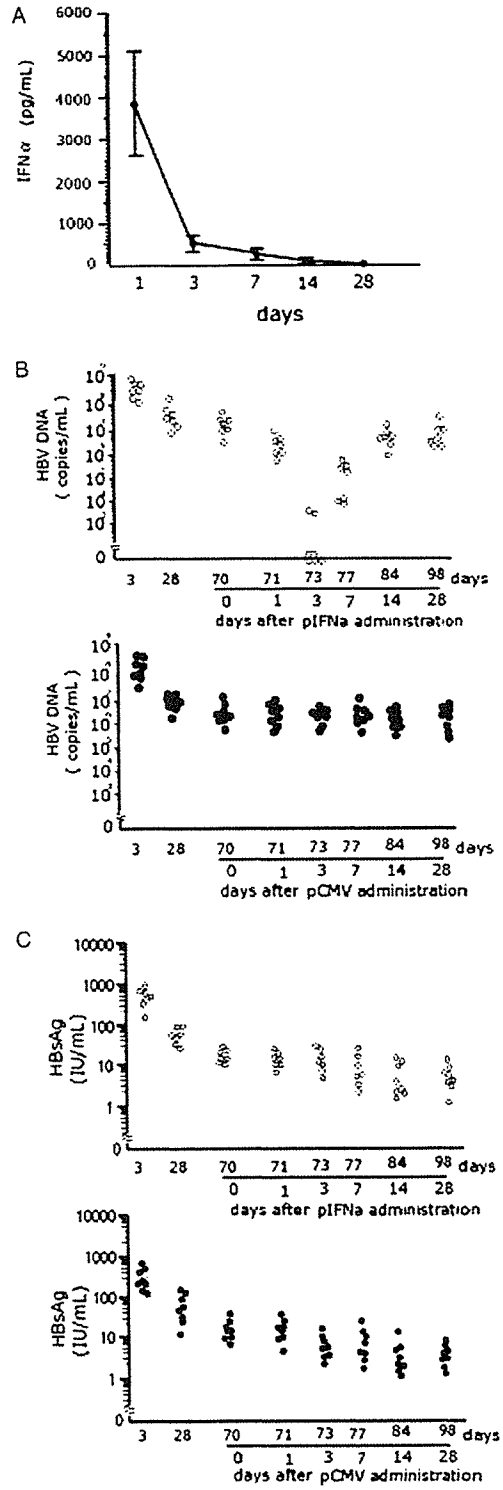

Fig. 6. Transient suppression of HBV production by IFNα gene therapy. (A) Serial determination of serum IFNα levels of nude mice after injection of pCMV-IFNa1. Horizontal bars indicate SD. (B and C) Nude mice were transfected with pHBV1.5 and, at 70 days later, transfected with either pCMV-IFNa1 (open circles) or pCMV (closed circles). Sera were serially obtained from the retro-orbital plexus, with HBV DNA (B) and HBsAg (C) titers being determined.

template as is the case of hepatitis C virus replicon systems [26,27]. Although HBV could not 'infect' murine hepatocytes, intracellular 'reinfection', namely recycling of HBV DNA occurs and leads to chronic viral production in the present model. Therefore, this model may provide a unique opportunity for analyzing possible mutations induced by long-term usage of various nucleotide analogs. Further study is needed to examine this possibility. Finally, intentional mutation could be easily introduced in inoculated DNA and a wide variety of mice with different genetic backgrounds can be used. The model presented here should enable analysis of viral as well as host factors that may regulate HBV replication.

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Quick Generation of Fully Mature Dendritic Cells From Monocytes With OK432, Low-Dose Prostanoid, and Interferon-α as Potent Immune Enhancers

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Summary: Dendritic cells (DCs) are one of the promising tools for enhancing antigen-specific immune responses in clinical settings. Many studies have been performed thus far to verify the efficacy of the DC vaccine in cancer patients; however, the responses have not always been satisfactory, partly because of DC incompetence. To obtain DCs potentially applicable for vaccination of cancer patients, our group sought to establish the strategy of DC generation mainly by modulating culture periods and maturation stimuli. Novel mature DCs that can be generated from monocytes within 3 days by using a combination of OK432 (*Streptococcus pyogenes* preparation), low-dose prostaglandin E_2 (PGE_2), and interferon-α (OPA-DCs) were developed. They strongly express CD83, CD86, and CCR7 and have potent ability to migrate to CCL21. In addition, they were able to activate natural killer and T helper 1 (T_H1) cells and to induce peptide-antigen–specific cytotoxic T lymphocytes more significantly than monocyte-derived DCs stimulated with a conventional cytokine cocktail of tumor necrosis factor-α, interleukin (IL)-1β, IL-6, and PGE_2 (monocyte-conditioned medium [MCM]-mimic DCs). The profound ability of OPA-DCs to stimulate these effectors is attributable to their higher expression of IL-12p70, IL-23, and IL-27 than MCM-mimic DCs, which was supported by the findings that the neutralization of IL-12p70 and IL-23 reduced the T_H1 priming ability of OPA-DCs. Even when from advanced gastric or colonic cancer patients, OPA-DCs displayed abilities of migration and T_H1 induction comparable to those from healthy subjects. Therefore, OPA-DCs may serve as a feasible vaccine with the potential to enhance T_H1-dominant and cytolytic immune responses against cancers.

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Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) that play a central role in innate and acquired immunity. For the treatment of cancers, tumor antigen-loaded DCs have been considered as a therapeutic vaccine to induce tumor-specific immunity. Many clinical studies have been performed to assess the efficacy of DC vaccine against cancers; however, favorable immunologic outcomes have been obtained from only half of the vaccinated patients.[1] Overall, the lessons from these studies are that mature DCs are better than immature ones to induce anticancer immune responses in the vaccinated patients.

Although the protocols of mature DC generation are yet to be standardized, a monocyte-conditioned medium (MCM)-mimic is widely used as a maturation stimulus for monocyte-derived DCs (MoDCs). The MCM-mimic is a combination of recombinant cytokines first reported by Jonuleit et al.[2] It gives rise to mature DCs in vitro; however, less than 10% of cancer patients vaccinated with MCM-mimic–treated DCs displayed favorable clinical responses (partial or complete remission).[3–5] These observations suggest that the MCM-mimic may fall short of generating mature DCs capable of inducing an in vivo immune response. One of the reasons may be that the MCM-mimic lacks the ability to promote DCs to secrete interleukin (IL)-12p70,[6] which is well known as an enhancer of cytotoxic activity of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs).[7,8] Therefore, it may be necessary to improve the maturation stimuli of DCs with respect to the functional requirements of the DC vaccine. From a mechanistic point of view, DCs loaded with antigens migrate into draining lymph nodes (DLs), where they activate NK cells or present antigens to $CD4^+$ and $CD8^+$ T cells.[9,10] Thus, to induce potent antitumor immunity, DCs need to possess the abilities to migrate and stimulate these effectors, which has been demonstrated in some murine models.[11–14]

Recently, other investigators have demonstrated that monocytes differentiate into mature DCs in 2 days when an

MCM-mimic is used.[15] It has also been reported that OK432, a penicillin-killed and lyophilized preparation of a low-virulence strain of *Streptococcus pyogenes*, provides MoDCs with the ability to induce a T helper 1 (T_H1) response.[16,17] In the present study, we sought to establish functionally mature MoDCs mainly by modulating culture periods and maturation stimuli. Using OK432 in combination with prostaglandin E_2 (PGE_2) and interferon (IFN)-α, we successfully generated mature DCs in only 3 days. In comparison with the MCM-mimic, this cocktail enables DCs to gain more potent abilities in migration, IL-12p70 secretion, NK cell activation, T_H1 induction, and CTL generation. These novel quickly generated DCs are a promising tool for developing DC vaccines against cancers.

MATERIALS AND METHODS

Reagents

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 were purchased from PeproTech (Rocky Hill, NJ). Recombinant human IL-7 and IL-2 were obtained from Genzyme-Techne (Minneapolis, MN). Recombinant human tumor necrosis factor (TNF)-α, IL-6, and IL-1β were purchased from R&D Systems (McKinley Place, NE). OK432 (Picibanil) was kindly provided by Chugai Pharmaceutical Company (Tokyo, Japan). The amount of OK432 is expressed in units designated as KE (Klinische Einheit [clinical unit]). One KE OK432 is equivalent to 0.1 mg dry streptococci. Natural human IFNα was kindly provided by Otsuka Pharmaceutical Company (Tokyo, Japan). PGE_2 was purchased from Sigma (St. Louis, MO). Nine-mer peptide carcinoembryonic antigen (CEA) 652(9) (TYACFVSNL), reported to be a human leukocyte antigen (HLA)-A24 restricted CTL epitope in CEA,[18] was purchased from TaKaRa Bio (Shiga, Japan).

Cell Lines

T2-A24 is a transporter associated with an antigen processing (TAP) deficient cell line (T2) transfected with HLA-A*2402 gene. This cell line expresses a high level of HLA-A24 protein and is used for targets in cytotoxicity assay (a kind gift from Dr. Hideaki Tahara, University of Tokyo, Tokyo, Japan). T2-A24 and NK cell-sensitive cell line K562 were cultured in RPMI-1640 containing 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2.

Preparation of Various Dendritic Cells From Monocytes

Buffy coat from healthy blood donors was kindly provided by the Red Cross Blood Center (Osaka, Japan). After written informed consent had been obtained, blood samples were collected from healthy donors or patients with primary untreated and advanced gastric or colonic cancer followed at Osaka University Hospital, Osaka Police Hospital, or Saiseikai Senri Hospital.

Peripheral blood mononuclear cells (PBMCs) were separated from the buffy coat or fresh peripheral blood from donors by standard density gradient centrifugation using Lymphocyte Separation Solution (Nacalai Tesque, Kyoto, Japan). Monocytes were isolated from PBMCs using anti-CD14 microbeads (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's instructions. The purity of the $CD14^+$ cells verified by flow cytometry was 90% to 95%.

Quickly Induced and Standard Immature Dendritic Cells

Monocytes were seeded on 24-well culture plates (Falcon, Franklin Lakes, NJ) at 5×10^5 per well and cultured in serum-free AIM-V media (Invitrogen, Carlsbad, CA) containing 50 ng/mL GM-CSF and 20 ng/mL IL-4. Nonadherent cells harvested on day 3 of culture were called quickly induced immature DCs (qiDCs). Half of the culture supernatants of the remaining cells were replaced with AIM-V media containing the same amount of GM-CSF and IL-4 on day 4. Subsequently, nonadherent cells of each group were harvested on day 7 and called standard immature DCs (siDCs).

Standard Mature Dendritic Cells

For the generation of standard mature DCs (smDCs), monocytes were cultured as for siDCs. During the final 24 hours, the cells were matured with 0.1 KE/mL OK432 in the presence (smDC-op) or absence (smDC-o) of 350 ng/mL PGE_2.

Quickly Induced Mature Dendritic Cells

For the generation of quickly induced mature DCs (qmDCs), monocytes were cultured as for qiDCs. During the final 24 hours, the cells were matured with 0.1 KE/mL OK432 in the presence (qmDC-op) or absence (qmDC-o) of various concentrations of PGE_2. In addition, other qmDCs were generated with some combinations of reagents or cytokines in a similar manner (combination of OK432, low-dose PGE_2, and IFNα [OPA]-DCs and MCM-mimic [MCMm]-DCs). The definitions of DCs generated in the various protocols are summarized in Figure 1.

Yield of Various Dendritic Cells

After the generation of various DCs, nonadherent cells were harvested and counted. Subsequently, the harvested cells were stained with anti-CD11c–fluorescein-isothiocyanate (FITC) monoclonal antibody (mAb) (KB90; DakoCytomation, Kyoto, Japan) and anti-HLA–D-related (DR)–phycoerythrin (PE) mAb (L243; Becton Dickinson, Franklin Lakes, NJ). The cells positive for CD11c and HLA-DR were defined as DCs. The absolute number of harvested various DCs was calculated from the percentage of DCs in the collected cells. The yield of DCs was defined as the percentage of recovered DCs in the seeded monocytes.

Analysis of Dendritic Cell Phenotype

DCs were analyzed for CD40, CD80, CD83, CD86, CCR7, CD14, and HLA-DR expression using fluorescent material–conjugated mouse mAbs. Anti-human CD40 mAb (5C3), anti-human CD80 mAb (L307.4), and anti-human CD83 mAb (HB15a) were from Immunotech (Marseille,

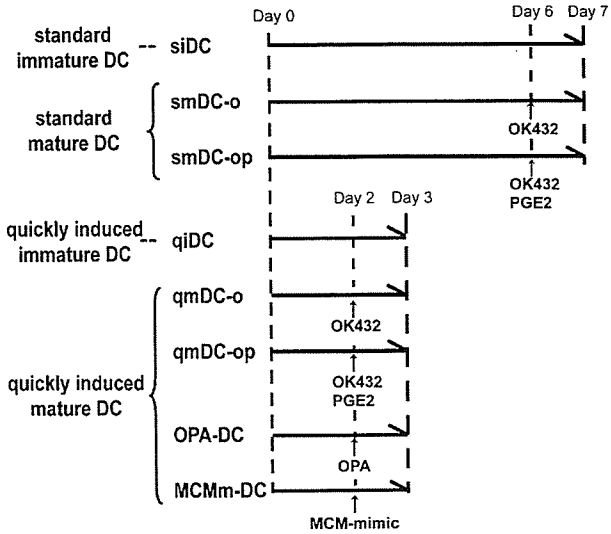

FIGURE 1. Preparation of DCs generated by means of various protocols. siDCs are generated with GM-CSF and IL-4 over 7 days. smDCs are siDCs stimulated with OK432 in the absence (smDC-o) or presence (smDC-op) of 350 ng/mL PGE_2 for the final 24 hours. qiDCs are generated from monocytes with IL-4 and GM-CSF for 3 days. qmDCs are qiDCs stimulated with OK432 combined with other reagents for the final 24 hours (qmDC-o: OK432 only, qmDC-op: OK432 and PGE_2). The concentrations of reagents used were 0.1 KE/mL OK432 and 10 to 1000 ng/mL PGE_2. In addition, qmDCs with OPA (OPA-DCs) or with MCM-mimic (MCMm-DCs) were generated for later experiments. (OPA: 0.1 KE/mL OK432, 500 IU/mL IFNα, and 50 ng/mL PGE_2; MCM-mimic: 10 ng/mL TNFα, 10 ng/mL IL-1β, 10 ng/mL IL-6, and 350 ng/mL PGE_2).

France). Anti-human CD86 mAb (BU63) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human CCR7 mAb (150503) was from R&D Systems. Anti HLA-DR mAb (L243) and anti-human CD14 mAb (MΦP9) were from Becton Dickinson. The phenotypic analysis of DCs was performed using FACSCaliber (Becton Dickinson) and Cell Quest software (Becton Dickinson).

Migration Assay

The migratory ability of DCs was examined by translocation of DCs in response to chemokine through a polycarbonate filter with 5-μm pores in 24-well transwell chambers (Corning Coster, Cambridge, MA). Into the lower chambers, 500 μL AIM-V with and without 500 ng/mL CCL21 (R&D Systems) was introduced. DCs were placed in the upper chambers at 1×10^5 per well and incubated for 2 hours at 37°C. The cells that migrated to the lower chambers were harvested and counted. The number of spontaneously migrated cells, those in the well without the addition of CCL21, was subtracted from the number of migrated cells in the well with the addition of CCL21.

Subsequently, the original cells before the migration assay and the cells migrating to the lower chamber containing CCL21 were stained with anti-CD11c-FITC mAb and anti HLA-DR-PE mAb, and the percentage of CD11c and HLA-DR double-positive cells was analyzed by flow cytometry. The percentage of migrated DCs in response to CCL21 was calculated as follows.

$$\text{Migrated DCs} = \frac{\%\text{ of double positive migrated cells} \times \text{migrated cells to CCL21}}{\%\text{ of double positive original cells} \times 1 \times 10^5} \times 100\,(\%)$$

Cytokine Measurement

Cytokine Producing Ability of Various Dendritic Cells

To test the ability of DCs to produce IL-12p70 and IL-10, DCs at a rate of 1×10^5 per well were cultured with 1×10^5 per well murine fibroblasts transfected with human CD40L (CD40L/L-cell) and 100 IU/mL IFNγ in 96-well flat-bottom plates (Asahi Techno Glass, Tokyo, Japan). After 24 hours of incubation, the culture supernatants were collected and the concentration of IL-12p70 and IL-10 in the samples was examined by means of enzyme-linked immunosorbent assay (ELISA).

Type 1 Helper T-Cell–Inducing Ability of Various Dendritic Cells

To test the ability of DCs to stimulate a T_H1 response, 1×10^5 per well allogeneic $CD4^+$ $CD45RO^-$ naive T cells were cultured with 1×10^4 per well DCs in 96-well flat-bottom plates. In some experiments, 10 ng/mL anti-human IL-23 polyclonal antibody and/or 25 ng/mL anti-human IL-12p70 mAb (24910; R&D Systems) was added to the culture for neutralization of each cytokine. Naive $CD4^+$ T cells were prepared from PBMCs by negative selection using a Stem-Sep system (StemCell Technologies, Vancouver, British Columbia, Canada) according to the manufacturer's instructions. The purity of isolated $CD4^+$ $CD45RO^-$ naive T cells was 90% to 95%, as determined by flow cytometry. On day 4, 10 ng/mL IL-2 was added to each well. On day 7, the cells were harvested and stimulated with 10 ng/mL phorbol-12-myristate-13-acetate (PMA; Sigma) and 1 μg/mL ionomycin (Sigma). After 24 hours of incubation, the culture supernatants were collected and the concentration of IFNγ and IL-10 in the samples was examined by means of ELISA.

Paired antibodies for the detection of human IL-12p70, IFNγ, and IL-10 were purchased from Endogen (Woburn, MA). The range of the assay was 15 to 1000 pg/mL.

Quantification of p19, p40, p28, and Epstein-Barr Virus–Induced Gene 3 Messenger RNA Expression of Various Dendritic Cells

Quantitative analysis of p19, p40, p28 and Epstein-Barr virus–induced gene 3 (EBI3) messenger RNA (mRNA) expression was performed in various DCs using real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from DCs using an RNeasy mini kit (Qiagen, Valencia, CA). First-strand complementary DNA (cDNA) was synthesized from the total RNA with the Super Script III First-Strand Synthesis System (Invitrogen).

Quantification of the p19, p40, p28, EBI3, and β-actin transcripts was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) according to the TaqMan probe method. The reaction protocol was identical for all PCR products. In brief, a 2-minute incubation at 50°C and a 10-minute incubation at 95°C were followed by 40 cycles of sequential incubations at 95°C (15 seconds) and 60°C (1 minute) for data collection. The β-actin mRNA expression of all samples was quantified as an endogenous standard, and normalization to the β-actin was performed for each sample.

Analysis of Natural Killer Stimulatory Activity of Various Dendritic Cells

The ability of DCs to stimulate autologous NK cells was assessed by the cytotoxicity of NK cells. NK cells were prepared from $CD14^-$ cells by positive selection using anti-CD56 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the isolated $CD56^+$ NK cells was 90% to 95%, as determined by flow cytometry. Various DCs were cultured with autologous NK cells in 24-well culture plates (Falcon) for 24 hours at 37°C in 5% CO_2. Subsequently, these cells were cultured with K562 cells labeled with $Na_2{}^{51}CrO_4$ at various effector/target (E/T) ratios for 4 hours at 37°C in 5% CO_2. For spontaneous release, target cells were incubated with medium alone, and for maximum release, target cells were incubated with medium containing 10% Triton X-100 (Sigma). Supernatants were then harvested, and radioactivity was counted with a Wizard 3 gamma counter (Wallac, Boston, MA). Percentages of target cell lysis were calculated as follows.

$$\text{Specific lysis} = \frac{\text{sample release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100\ (\%)$$

Induction of Carcinoembryonic Antigen–Peptide–Specific Cytotoxic T Cells With Various Dendritic Cells

CTLs specific for CEA 652(9) peptide were generated according to a method described previously.[19] After informed consent had been obtained, fresh blood samples were taken from HLA-A24–positive healthy donors. As for responder cells, $CD14^-$, $CD19^-$, and $CD56^-$ cells were isolated by depleting $CD19^+$ and $CD56^+$ cells from $CD14^-$ cells using anti-CD19 and anti-CD56 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. After various DCs were generated as described previously, they were incubated with 20 μg/mL CEA.652(9) peptide for 6 hours at 37°C. Subsequently, they were cultured with autologous $CD14^-$, $CD19^-$, and $CD56^-$ cells in DC medium (DCM) for 28 days at 37°C in 5% CO_2. The DCM is the Iscove modified Dulbecco medium (Invitrogen) containing 10% FCS, 2 mM L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Invitrogen), 100 μM nonessential amino acid (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin. On days 7, 14, and 21, the same numbers of peptide-loaded freshly prepared DCs from the same donor were supplied to the culture as stimulators. Recombinant human IL-7 was added at 5 ng/mL on days 1 and 7. In addition, recombinant human IL-2 was added at 5 ng/mL every 3 days from day 10 of CTL induction.

Analysis of Carcinoembryonic Antigen–Peptide–Specific Cytotoxic T-Cell Activity

CEA-peptide–specific cytotoxic T cells were induced with various DCs as mentioned previously. On day 28 of CTL induction, the cells were harvested. Before analysis of cytotoxicity, the remaining $CD56^+$ cells were removed from the DC-primed cells with anti-CD56 microbeads (Miltenyi Biotec). The lytic activity of CTL against CEA 652(9) peptide-loaded T2-A24 was assessed by means of a ^{51}Cr releasing assay. To confirm that the lytic activity is exerted in an HLA class I-restricted and CD8-restricted manner, 10 μg/mL mouse monoclonal anti-HLA–ABC antibody (W6/32) (Serotec, Oxford, UK) or mouse monoclonal anti-human CD8 antibody (DK25) (DakoCytomation) was added to the mixture of effectors and targets. To exclude the possibility of the lytic activity being mediated by NK cells, K562 was also used as a target cell in the assay.

Statistical Analysis

The results are expressed as the mean ± standard error of the mean (SEM). Statistical significance was analyzed using the paired 2-tailed *t* test with Prism 4 software (GraphPad Software, San Diego, CA). A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Mature Dendritic Cells Can Be Quickly Induced by OK432

Other investigators have reported that monocytes differentiate into DCs even within 48 hours.[15] Quick generation of DCs is beneficial for the maintenance of their cell viability. Thus, to examine whether OK432 can induce mature DCs in a shorter culture period, we added it to the culture on day 2. Although all DCs were positive for HLA-DR but negative for CD14, qiDCs expressed lesser degrees of CD80, CD83, and CD86 than siDCs. OK432 enhanced the expression of costimulatory factors (CD40, CD80, CD83, and CD86) on qmDC-o as well as on smDC-o, however (Fig. 2). Thus, the addition of OK432 to DCs at an early phase induced phenotypically mature DCs in a short culture period.

Combination of OK432 and Prostaglandin E_2 Quickly Induces Mature Dendritic Cells With Potent Migratory Ability

To compare the migratory capability of DCs generated by quick or standard generation protocols, we examined CCR7 expression and the migration of DCs to CCL21 in a transwell